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Isolation and partial characterization of a low molecular weight antimicrobial protein from coconut (*Cocos nucifera* L.) milk

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Abstract

A low molecular weight protein from coconut (*Cocos nucifera* L.) milk was extracted using phosphate buffer solution pH 7.6, ammonium sulfate precipitation, and dialysis. The insoluble fraction had higher protein concentration (22.39 µg/mL) than the soluble fraction (9.95 µg/mL) and the electrophoretic profile on SDS-PAGE also showed that the low molecular weight proteins were concentrated in the insoluble fraction. The fraction containing the low molecular weight proteins were purified using DEAE-Cellulose ion-exchange chromatography followed by gel filtration chromatography using Sephadex G-75. The isolated protein has an estimated molecular weight of 19kDa using the electrophoretic mobility on SDS-PAGE and on the elution parameter by gel filtration chromatography. The low molecular weight protein showed antimicrobial properties against food spoilage yeasts, *Debaryomyces hansenii* and *Candida albicans*.

Introduction

Plant proteins have shown protective properties against various types of infectious agents and has exhibited several health benefits such as immunostimulating (Tsuruki *et al*., 2003), antihypertensive (Kuba *et al*., 2005; Kodera and Nio, 2006), and hypocholesterolemic properties (Zhong *et al*., 2007). These plant proteins with varied physiological and biological functions can be classified based on its solubility and molecular weight (Tandang, 2000). The high molecular weight proteins are usually the major storage proteins and are the fractions present in high amounts as compared to low molecular weight protein fractions. But there is a growing interest in low molecular weight proteins of plant origin due to the fact that many have antimicrobial and carcinostatic actions (Salakhutdinov *et al*., 1998)

Among the many plant sources, coconuts (*Cocos nucifera* L.) provide a good potential source of proteins with good nutritional value and a relatively well-balanced amino acid profile. Coconut milk is considered as a valuable food for people having nutritional deficiencies since it has significant amount of Vitamin C, adequate natural minerals, and a high quality protein. But as yet, very little are still known about the coconut proteins as studies are focused on its high oil content which has also showed many health benefits (Carandang, 2008).

Various fractionation as well as purification and characterization studies on coconut have been made. Sjogren and Spychalski (1930) have isolated cocosin, a high molecular weight protein, from the endosperm; Kwon and colleagues (1996) have fractionated and characterized five protein fractions from coconut flour; and Garcia and colleagues (2005) have isolated and characterized total globulins of the mature coconut endosperm.

Aside from the nutritional properties, a wide number of proteinaceous compounds have also been found to have deleterious effects toward microorganisms and these are known as antimicrobial peptides (Mandal *et al*., 2009). Research on antimicrobial proteins and peptides has attracted the attention of a large number of investigators to find novel approaches to control infectious and pathogenic microorganisms. There have been a few reports that showed the presence of antimicrobial peptides in coconut, Wang and Ng (2002) have isolated a peptide from coconut meat with antifungal activity against *Fusarium oxysporum, Mycosphaerella arachidicola,* and *Physalospora piricola*. In another study, Mandal *et al.* (2009) have identified from green coconut water three antibacterial peptides against Gram-positive and Gram-negative bacteria including *Escherichia coli, Bacillus subtilis, Staphylococcus aureus*, and *Psuedomonas aeruginosa*. It is therefore important to determine the presence of low molecular weight antimicrobial proteins in coconut milk against

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common food spoilage microorganisms.

Materials and Methods

Sample preparation

Mature coconut used in this study was obtained from the local market of Los Baños, Laguna. The coconut endosperm was grated, wrapped in cheesecloth, and then squeezed using a fabricated press to collect the milk. The collected coconut milk was filtered using cheesecloth, freeze dried, placed in sealed containers, and kept in the freezer until used.

Protein extraction

The extraction of coconut proteins was done following the modified method outlined by Garcia *et al.* (2005). The freeze dried coconut milk was defatted for 1 hr using n-hexane (1:10 w/v) twice then dried under the fumehood. Protein extraction was done for 1 hr with shaking using phosphate buffer (35 mM potassium phosphate buffer, pH 7.6 with 0.4M NaCl, 0.1 mM PMSF, 10mM β-mercaptoethanol, 0.02% sodium azide, 1 mM EDTA). The mixture was filtered and centrifuged at 3000 rpm for 30 mins. The supernatant was collected and added with ground ammonium sulfate until 80% saturation, placed in shaker for 1 hr in an ice bath, and centrifuged at 3000 rpm for 30 mins. The precipitate was collected, dissolved in minimum amount of phosphate buffer, and dialyzed for 24 hrs with four changes in an iced bath. The dialysate was centrifuged to separate the soluble and insoluble fractions and kept in the freezer for further analysis. Both fractions were subjected to Bradford analysis and SDS-PAGE to determine in which fraction was the low molecular weight proteins concentrated.

Protein content determination

The protein content of the samples during purification was determined according to the method of Bradford (1976) using Bovine Serum Albumin (BSA) as the protein standard.

SDS-PAGE

SDS-PAGE was carried out by the method of Laemmli and Favre (1970) using 12% acrylamide. Obtained gel from electrophoresis was stained with 0.25% Coomassie-Blue R-250 for 30 mins followed by de-staining using 40% methanol-7% acetic acid in water solution. The molecular weight of the sub-units was estimated by using BenchMarkTM Protein Ladder.

Ion- exchange chromatography

Aliquots of proteins dissolved in 35 mM phosphate buffer, pH 7.6, were applied to a DEAE-cellulose column (2 × 37 cm) equilibrated with the same phosphate buffer. The elution was initially carried out using the phosphate buffer and further elution was carried using a discontinuous gradient of NaCl from 0 to 1.0 M in the same buffer. Fractions of 3 mL were collected at a constant flow rate of 0.5 ml/min and the absorbance was monitored at 280 nm using Shimadzu UV Mini-1240 spectrophotometer.

Gel filtration chromatography

Gel filtration chromatography was used to determine the molecular weight of the protein obtained. A column (2×55 cm) of Sephadex G-75 was used, previously equilibrated with the eluting solvent. The calibration kit used consisted of ribonuclease A (13,700 Da), chymotrypsinogen A (25,000 Da), ovalbumin (43,000 Da), and bovine serum albumin (67,000 Da) prepared according to the kit manual. The void volume was determined with blue dextran.

Antimicrobial activity assay

The fraction containing the low molecular weight protein from gel filtration chromatography was assayed for antimicrobial activity toward *Debaryomyces hansenii, Candida albicans, Aspergillus niger,* and *Bacillus cereus* using the Kirby-Bauer disk diffusion susceptibility test in 100 mm × 15 mm Petri plates. Potato dextrose agar (PDA) was used for *Debaryomyces hansenii, Candida albicans,* and *Aspergillus niger* while Nutrient agar (NA) was used for *Bacillus cereus*. The plates were incubated at 30°C for two days for the *Debaryomyces hansenii, Candida albicans* and *Bacillus cereus* while three days for the *Aspergillus niger*.

Results

Protein extraction

Bradford Assay revealed that the insoluble fraction has higher protein content (22.39 µg/mL) than the soluble fraction (9.95 µg/mL). From the electrophoretic profile (Figure 1) of both fractions, it was observed that the insoluble fraction contains mostly the high molecular weight and low molecular weight proteins as seen by the major protein bands ranging from 14 kDa to 111 kDa while the soluble fraction contains most of the higher molecular weight proteins ranging from 50 kDa to 126 kDa. The molecular weight values obtained from both fractions were within the reported molecular weight values of the coconut milk proteins in a study conducted by

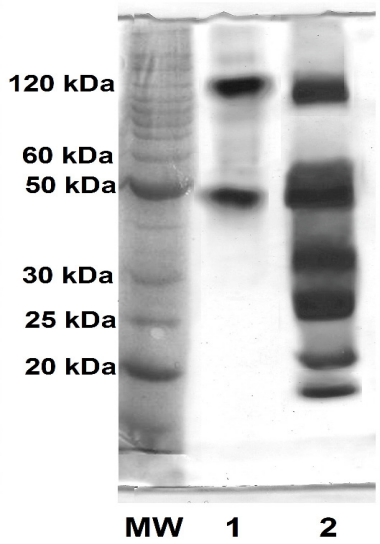

Figure 1. SDS-PAGE profile of proteins extracted from coconut water. MW is the molecular weight marker; 1 is the soluble fraction; and 2 is the insoluble fraction

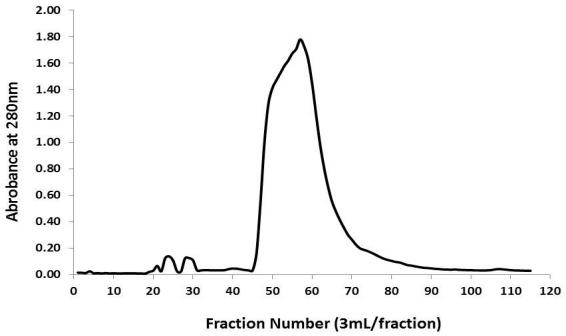

Figure 2. Ion-exchange chromatography elution profile of the insoluble protein fraction in DEAE-Cellulose column

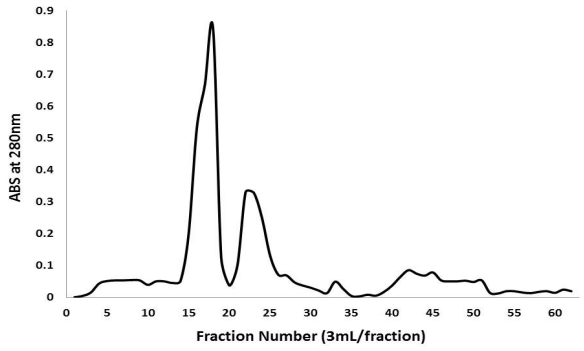

Figure 3. Gel filtration elution profile on a 2×50 cm Sephadex G-75 column. Peak I, high molecular weight proteins; peak II, low molecular weight proteins

Sumual (1994) which ranges from 8 kDa to 209 kDa.

Ion-exchange chromatography

The absorbance readings of the unbound proteins were measured to be less than 0.2 and produced small peaks (Figure 2). These were considered as

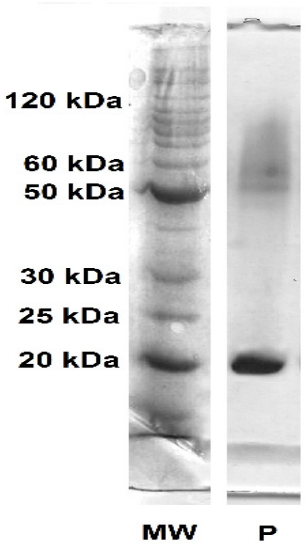

Figure 4. SDS-PAGE profile of the low molecular weight protein (P) from Sephadex G-75

the neutral molecules having the same charge as the matrix thus there is no interaction and are just eluted. The bound proteins were eluted using a continuous linear gradient of 0 to 1 M NaCl and the absorbance readings of the fractions 48 to 70 reached up to 1.78 at 280 nm which produced one large peak and are the fractions containing the negatively charged proteins.

Gel filtration chromatography

The elution pattern (Figure 3) of the protein sample resulted to two peaks. The Peak 1 was mostly composed of high molecular weight proteins (55kDa) while Peak 2 was composed of low molecular weight proteins (19 kDa) as calculated from the calibration curve established from the protein kit standards. When the protein fraction under peak 2 was subjected to SDS-PAGE, it showed only one sub-unit also with an estimated molecular weight of 19 kDa computed using its electrophoretic mobility in comparison to the molecular weight marker as showed in Figure 4. This suggested that the isolated low molecular weight protein is a monomer since it did not break further into sub-unit constituents. The estimated molecular weight from SDS-PAGE was in agreement with the estimated molecular weight calculated from the calibration curve of protein kit standards obtained in gel filtration chromatography.

Antimicrobial activity of the low molecular weight protein

After the specified incubation time for each microorganism, it was observed that the low molecular protein was effective at 2.5 µg/µL against the yeasts *Debaryomyces hansenii* and *Candida*

Table 1. Zone of inhibition of the four test microorganisms against the isolated low molecular weight protein

Microorganism	Ave. Diameter of Zone of Inhibition, mm	
	1.5µg/µL	2.5µg/µL
Candida albicans	8	10
Debaryomyces hansenii	8	12
Aspergillus niger	0	7
Bacillus cereus	0	0

albicans as shown by the larger diameter zone of inhibition created. It has minimal effect to *Aspergillus niger,* and no effect on *Bacillus cereus*. Table 1 shows the measured zone of inhibition created by the test microorganisms.

The isolated low molecular weight protein has an intermediate effect on the yeasts *Debaryomyces hansenii* and *Candida albicans* while *Aspergillus niger* and *Bacillus cereus* were resistant. Based on the performance standards for antimicrobial susceptibility testing established by the Clinical and Laboratory Standards Institute (CSLI), an intermediate effect means that there is attainable efficacy and a higher dosage or concentration may be used to increase the susceptibility of the microorganism and make the antimicrobial agent more effective. Resistant effect implies that the microorganisms are not inhibited by the usually achievable concentrations of the antimicrobial agent or the microorganisms have a specific resistance mechanism.

Conclusion

The isolated protein extracted from freeze-dried coconut milk was identified to be a negatively charged monomer with a molecular weight of 19 kDa and showed antimicrobial activity when tested against *Debaryomyces hansenii* and *Candida albicans*. This result indicates that aside from the nutritional benefits of coconut milk proteins, it has other functional properties and has a potential role in inhibiting food spoilage yeasts.

Acknowledgement

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